

## Ovine dendritic cells transduced with an adenoviral CTLA4<sub>e</sub>EGFP fusion protein construct induce hyporesponsiveness to allostimulation

ASHLEY NEWLAND,\*† SVJETLANA KIRETA,\* GRAEME RUSS\*† & RAVI KRISHNAN\*† \**Transplantation Immunology Laboratory, Basil Hetzel Institute, The Queen Elizabeth Hospital, Woodville and †Department of Medicine, University of Adelaide, South Australia, Australia*

### SUMMARY

CTLA4 (CD152) is a transmembrane molecule expressed on activated T cells and functions as a negative regulator of T cell activation upon binding to the costimulatory molecules CD80/86. In this study, CTLA4<sub>e</sub>EGFP constructs were engineered by cloning the extracellular domains of ovine and human CTLA4 (CTLA4<sub>e</sub>) 'in frame' with the enhanced green fluorescent protein (EGFP). Recombinant adenoviral vectors were generated by incorporation of the CTLA4<sub>e</sub>EGFP sequence into the adenoviral genome using homologous recombination in *Escherichia coli*. The functional activity of the adenoviral vectors was shown by the secretion of the CTLA4<sub>e</sub>EGFP upon infection of ovine fibroblasts and the binding of the fusion protein to the target ovine and human dendritic cells expressing CD80/86 receptors by flow cytometry. The EGFP tag facilitated molecular size determinations and quantification of the secreted ovine CTLA4 fusion protein by immunoprecipitation and enzyme-linked immunosorbent assay (ELISA), respectively, using anti-GFP mAbs. Ovine dendritic cells obtained from pseudo-afferent lymphatic cannulation of sheep were characterized based on high major histocompatibility complex (MHC) class II expression and cross-reactivity with monoclonal antibodies to the human dendritic cell markers, CD83 and CMRF-56. In addition, ovine dendritic cells (DC) were transfected with the adenoviral CTLA4<sub>e</sub>EGFP and when used as stimulators in a mixed lymphocyte reaction showed a reduced capacity to induce allogeneic lymphocyte proliferation. This study verifies that the ovine CTLA4<sub>e</sub>EGFP fusion protein functions similarly to its human homologue and that DC modified with adenoviral CTLA4-EGFP may provide an effective therapeutic approach in targeting alloreactive T cells to prolong allograft acceptance in a preclinical ovine model of renal transplantation.

**Keywords** adenovirus, CTLA4, green fluorescent protein, MLR, sheep

### INTRODUCTION

The interaction of the costimulatory molecule CD28 on T cells with its receptors, CD80/86 on antigen-presenting cells results in T cell activation.<sup>1</sup> However, the control of T

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Abbreviations: DC, dendritic cells; EGFP, enhanced green fluorescent protein; PBMC, peripheral blood mononuclear cells; CsA, cyclosporin A; *ov*-, ovine; *hu*-, human.

Correspondence: Dr Ravi Krishnan, Transplantation Immunology Laboratory, Basil Hetzel Institute, The Queen Elizabeth Hospital, 28 Woodville Road, Woodville, South Australia, Australia 5011. E-mail: ravi.krishnan@adelaide.edu.au

cell activation is regulated by CTLA4, a structural homologue of CD28 which is expressed on activated T cells.<sup>2,3</sup> The binding of CTLA4 to CD80/86 inhibits T cell proliferation by down-regulation of interleukin (IL)-2 production and blockade at the G1 phase of the cell cycle.<sup>4,5</sup> In addition, the stronger affinity of CTLA4 to its receptors<sup>6</sup> has been exploited experimentally to generate soluble CTLA4-Ig fusion proteins, which serve as costimulatory blockade agents by competing with CD28.<sup>4</sup>

Dendritic cells (DC) are the major antigen-presenting cells that initiate rejection against organ transplants. Genetic modification of donor DC is a possible means of inducing allospecific tolerance as these cells express donor antigen/major histocompatibility complexes (MHC) and migrate readily to lymphoid tissues. The pretransplant

administration of immature DC, with low costimulatory molecule expression of CD80/86, shows only a modest prolongation of allograft survival<sup>7</sup> because these dendritic cells mature subsequently *in vivo* to express high levels of costimulatory molecules. However, attempts to inhibit DC maturation have been assessed by genetic modification with immunomodulatory molecules. The cytokines IL-10 and transforming growth factor (TGF)- $\beta$  are able to down-regulate the expression of costimulatory molecules including the CD80/86 ligands, arresting DC in an immature state.<sup>8,9</sup> There is accruing evidence that genetic manipulation of DC to express TGF- $\beta$ <sup>10</sup> and IL-10<sup>11</sup> results in the inhibition of rejection. Moreover, the approach of blocking the CD80/86 ligands using CTLA4-Ig has shown improved allograft survival in an islet transplant model in mice by the use of a DC cell-line modified genetically with an adenoviral CTLA4-Ig vector.<sup>12</sup> To date reports on the use of genetically modified DC in large animal models of allotransplantation are not forthcoming.

This study validates the *in vitro* effects of ovine DC transfected with an adenovirus encoding a novel ovine CTLA4<sub>e</sub>EGFP fusion protein construct. Ovine DC were obtained by the cannulation of the prefemoral lymphatic vessel of sheep. DC phenotype and function was confirmed by strong expression of the CD83 and MHC class II markers and the potency to stimulate allogeneic ovine lymphocyte proliferation.

The EGFP tag of ovine CTLA4<sub>e</sub>EGFP allowed the real-time visualization of transfected cells by fluorescent microscopy, while the use of anti-GFP mAbs permitted molecular size determinations of the fusion protein on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) by immunoprecipitation and quantitation by enzyme-linked immunosorbent assay (ELISA) despite the lack of ovine specific mAbs for CTLA4. Moreover, high levels of transfection of DC with adenoviral vectors was achieved by the technique of combining the cationic liposome, lipofectamine, together with adenoviral particles to optimize gene transduction. Thus genetically modified ovine dendritic cells with alloinhibitory characteristics may be examined in our established preclinical ovine model of renal allograft transplantation.<sup>13</sup>

## MATERIAL AND METHODS

### *Cloning of the extracellular domain of ovine and human CTLA4*

Peripheral blood mononuclear cells (PBMC) were isolated from ovine and human peripheral blood by density gradient separation using Lymphoprep<sup>TM</sup> (Nycomed, Norway) and resuspended in complete medium at  $1 \times 10^6$  cells/ml. Human buffy coats were obtained from healthy blood donors (Australian Red Cross Transfusion Service, Adelaide, Australia). Total RNA was extracted from PBMC stimulated with  $1 \mu\text{g/ml}$  Con A FOR 48 hr by the established method of Chomczynski and Sacchi.<sup>14</sup> Reverse transcription was performed with MMLV reverse transcriptase (GibcoBRL, USA) on  $1 \mu\text{g}$  of total RNA. The extracellular domains of human and ovine CTLA4 were polymerase

chain reaction (PCR) amplified using Tth polymerase (Fisher Biotech, USA) for 26 cycles of 30 seconds at  $94^\circ$ , 30 seconds at  $55^\circ$  and 30 seconds at  $72^\circ$ , followed by a final extension at  $72^\circ$  for 7 min. OvCTLA4<sub>e</sub> PCR was performed using forward (5'-GGGAGATCTATGGCTTGCTCTGGATTCCAGAGTC-3') and reverse (5'-GAGGTACCGAATCCGGGCATGGTTCTGGA-3') primers based on the published sequence<sup>15</sup> (GenBank Accession number: AF092740). HuCTLA4<sub>e</sub> PCR was performed using forward (5'-GGGAGATCTATGGCTTGCCTTGGATTTCAGCGC-3') and reverse (5'-GGGGTACCGAATCTGGGCA CGTTCTGGATCA-3') primers based on the published sequence<sup>16</sup> (GenBank Accession number: NM\_005214). *Bgl*II and *Kpn*I restriction sites (underlined) were engineered into the forward and reverse primers, respectively. PCR products were digested with *Bgl*II and *Kpn*I and cloned 'in frame' with the EGFP gene in a pEGFP-N1 expression vector (Clontech, USA). The CTLA4 insert in the resulting plasmid vector constructs povCTLA4<sub>e</sub>EGFP and phuCTLA4<sub>e</sub>EGFP were confirmed to be authentic by sequencing and GenBank database analysis.

### *Generation of adenoviral CTLA4<sub>e</sub>EGFP constructs*

Adenoviral constructs containing the ovine and human CTLA4<sub>e</sub>EGFP (ADVovCTLA4<sub>e</sub>EGFP and ADVhuCTLA4<sub>e</sub>EGFP, respectively) and the EGFP-vector blank (ADV-EGFP) were generated using protocols established by He.<sup>17</sup> Ovine and human CTLA4<sub>e</sub>EGFP was excised from povCTLA4<sub>e</sub>EGFP and phuCTLA4<sub>e</sub>EGFP, respectively, by *Bgl*III/*Not*I digestion and ligated into the pShuttleCMV vector (Stratagene, USA). Homologous recombination was achieved by coelectroporating *Pme*I linearized recombinant pShuttleCMV vector with the pAdEasy1 vector into BJ5183 cells. pAdEasy1 vector is a replication-deficient adenovirus containing the genome for the AD 5-strain that was kindly provided by Dr B. Vogelstein, Howard Hughes Medical Institute. In order to generate recombinant adenoviral particles, the adenoviral constructs, ADVovCTLA4<sub>e</sub>EGFP, ADVhuCTLA4<sub>e</sub>EGFP and ADV-EGFP were linearized with *Pac*I and used to transfect the HEK-293 packaging cell line with Lipofectamine (GibcoBRL, USA). Infectious adenoviral particles were obtained by freeze–thaw–lysis of the transfected HEK-293 cells and purified by CsCl density gradient ultracentrifugation.<sup>18</sup> Quantification of adenoviral titres was performed by the cytopathic effect assay<sup>19</sup> and values expressed as plaque forming units (pfu).

### *Immunoprecipitation*

Gene transfer was conducted by electroporation of Chinese hamster ovary (CHO) cells with plasmid constructs and infection of fibroblasts with recombinant adenovirus. After overnight incubation, cells were cultured in methionine/cysteine free RPMI (ICN, USA) supplemented with glutamine and 1% fetal calf serum (FCS) and incubated at  $37^\circ$  for 1 hr. Media was replaced with 1.5 ml methionine/cysteine free media containing  $100 \mu\text{Ci/ml}$  Tran<sup>35</sup>S-Label<sup>TM</sup> (ICN Biomedicals, CA) and incubated overnight. Conditioned media were treated with 10% (v/v)

protease inhibitor and blocked with 5% (v/v) normal rabbit serum. Media was precleared with *Staphylococcus aureus* in RIPA buffer (1M NaCl, 10% sodium deoxycholate, 10% SDS, 1% NP40, 1M TRIS-HCL) at 11 600 g for 5 min at 4°. Supernatant was treated with 5 µg mouse anti-GFP mAb (Roche, USA) and incubated at 4° rotating overnight. *S. aureus* supplemented with 1 mg/ml ovalbumin (100 µl) was added for 1 hr and washed twice with RIPA buffer at 3870 g. Pellets were resuspended in Laemmli's buffer with an aliquot reduced with β-mercaptoethanol and after incubation at 100° for 15 min samples were run on 12.5% SDS-PAGE gels.<sup>20</sup> Gels were stained with coomassie blue and impregnated with Amplify™ (Amersham, UK) for 30 min prior to autoradiography on Kodak XAR5 film.

#### *Preparation of ovine and human CTLA4<sub>e</sub>EGFP fusion protein*

Ovine adult skin fibroblasts were infected with adenoviral particles at a multiplicity of infection (MOI) of 50. Adenoviral constructs consisted of either ov- or hu-CTLA4<sub>e</sub>EGFP and an EGFP vector blank control. Cells were incubated with the appropriate recombinant adenoviral vector for 1 hr to permit viral adsorption followed by the addition of fresh medium and incubation for a further 48 hr to allow for gene transduction. Conditioned medium was obtained by centrifugation of the cultures.

#### *Quantification of EGFP and CTLA4<sub>e</sub>EGFP fusion protein by ELISA*

An ELISA was established to quantify the CTLA4<sub>e</sub>EGFP secreted from adenoviral infected ovine fibroblasts. Due to the unavailability of ovine CTLA4 specific antibodies, capture and detection antibodies were directed against the EGFP fusion protein. Titertek Immuno Assay Plates (Flow Laboratories, Netherlands) were coated with polyclonal goat anti-GFP antibody (Rockland, USA) at 2 µg/ml in bicarbonate buffer (pH 9.6) for 2 hr at room temperature and then incubated overnight at 4°. Standard curves were generated by titrating recombinant EGFP (Clontech, USA) in the antibody-coated plates at a concentration range of 0.001–10 ng/ml. Serial dilutions of the unknown concentrations of EGFP and CTLA4<sub>e</sub>EGFP in conditioned medium were also added to the antibody-coated plates. After overnight incubations, and following washes with phosphate buffered saline (PBS) Tween-20, 1 µg/ml of mouse anti-green fluorescent protein (GFP) mAb was used to detect the captured fusion protein and native EGFP for 2 hr at room temperature. Then a 1/1000 dilution of the secondary biotinylated horse anti-mouse IgG conjugate (Vector, USA) in 10% FCS was added to the plates and was detected with a 1/1000 dilution of streptavidin-alkaline-phosphatase (Roche, USA) in PBS/Tween-20. The colorimetric reaction was produced by the addition of Sigma 104 phosphatase substrate (Sigma, USA) in diethanolamine buffer and absorbance at A<sub>405</sub> was determined after terminating the reaction with 0.5M

EDTA. Because the standard curve was derived with native EGFP the values obtained for CTLA4<sub>e</sub>EGFP depict equivalent molar concentration.

#### *Preparation of dendritic cells from human and ovine sources*

(i) *Human monocyte-derived DC.* Human monocytes were obtained by adherence of 5 × 10<sup>7</sup> PBMC in 75 cm<sup>2</sup> plastic tissue culture flasks (Corning, USA). After the removal of non-adherent cells, the monocytes were first differentiated into immature DC in complete medium supplemented with 400 U/ml IL-4 (Peprotech, USA) and 800 U/ml granulocyte macrophage-colony stimulating factor (GM-CSF) (Kenilworth, USA) for 5 days and then matured by the addition of 10 ng/ml tumour necrosis factor (TNF)-α (Genzyme Corporation, USA) for a further 2 days.<sup>21</sup>

(ii) *Ovine lymphatic DC.* Ovine lymphatic cells were collected by cannulation of the pseudoafferent lymphatic vessels from 2-year-old ewes as described previously by Dandie.<sup>22</sup> The prefemoral lymph nodes were excised surgically and after a period of 6–8 weeks of agistment the lymphatic vessels were identified by subcutaneous administration of 15 mg methylene blue (Pharmalab, Australia) and cannulated with a CBAS™ heparin-coated catheter (Carmeda, USA). The cannula was externalized and drained into a 100-ml sterile bag containing 2500 IU heparin and 40 mg gentamycin. Approval for these surgical procedures was obtained from the TQEH institutional animal ethics committee. DC were enriched on a 14% (w/v) metrizamide (Sigma, USA) density gradient and characterized by flow cytometry.

#### *Flow cytometry*

(a) *Ovine DC phenotypic analysis.* Cell-surface phenotype of the human monocyte-derived DC and the metrizamide-enriched ovine lymphatic DC was analysed by flow cytometry. Monoclonal antibodies (mAbs) were either against ovine cell-surface antigens or were mAbs known to cross-react with their ovine homologue. Briefly, cells were washed in PBS containing 1% FCS and 0.1% sodium azide in FACS wash buffer and incubated for 30 min on ice with 50 µl of either saturated supernatant or 1–2 µg/ml purified primary antibody. After two washes in FACS wash buffer, cells were incubated for 30 min on ice with isotype-specific, fluorescein isothiocyanate (FITC)-conjugated, anti-mouse antibodies and fixed in FACS lysing solution (BD Biosciences, USA). Isotype-control antibodies were used to determine background staining. MAb used were: αCD1a (SBUT6), αCD4 (44-38), αCD8 (38-65), αCD25 (9-14), αCD44 (25-32), Class I (41-19), Class II DP (28-1) obtained from the Centre of Animal Biotechnology, School of Veterinary Science, University of Melbourne. Other antibodies included αCD14 (VPM65), αCD83 (HB15A17-11), αCD31 (CO.3E1D4) and αCD21 (CC21) from Serotec, USA. CMRF56 antibody was obtained from Dr D. Hart, Mater Medical Research Institute, Brisbane, and 1D4-5 from Dr P. Hart, Flinders University of South Australia and X63 (P3 X63Ag8) from ATCC, USA.

(b) *CTLA4<sub>e</sub>EGFP binding activity.* Ovine and human DC were used as target cells to establish the binding activity of ovCTLA4<sub>e</sub>EGFP. Primary mAbs directed against the CD80 (Immunotech, France) and CD86 (Serotech, UK) ligands were used to confirm the expression of these molecules on the human DC. The CTLA4<sub>e</sub>EGFP binding assay involved three steps. DC were incubated first with 200 µl of ovCTLA4<sub>e</sub>EGFP at a concentration of 20 µg/ml. Secondly, cells were washed and incubated with a primary anti-GFP (IgG1) mAb (Roche, USA). X63 was used as an IgG1-isotype-matched negative control mAb. Finally, a polyclonal anti-mouse Ig Fab2 FITC-conjugated antibody (Silenus, Australia) was added to the cells to detect the primary antibody. Human CTLA4Ig (4 µg, R&D Systems, USA) was added to both human and ovine DC prior to the addition of ovCTLA4<sub>e</sub>EGFP in the binding assay to establish the specificity of binding to CD80/86. Flow cytometric analysis was performed using a Becton Dickinson FACScan.

#### Two-way mixed lymphocyte reaction

Conditioned media from recombinant adenoviral infected fibroblasts were quantitated by ELISA and tested for inhibitory activity in a two-way mixed lymphocyte/leucocyte reaction (MLR) as described below. Either sheep or human PBMC from two unrelated donors were cocultured in 96-well round-bottomed plates to a final cell concentration of  $2 \times 10^5$  cells per well. The MLR was performed in complete medium in a volume of 200 µl. The mixed cultures were incubated at 37° for 4 days and then pulsed for 20 hr with 1 µCi of [<sup>3</sup>H]thymidine. The cells were harvested onto filters and the incorporated radioactivity was determined by liquid scintillation counting in a Wallac Microbeta Counter (Turku, Finland). All determinations were expressed as counts per minute (cpm) and performed in triplicate.

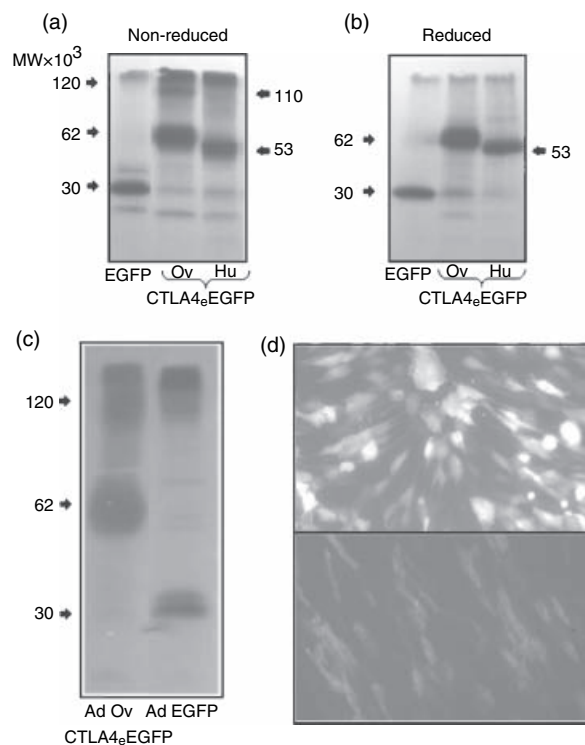
#### Genetically modified DC as stimulators in the MLR

Unlike gene transfer by infection of fibroblasts, DC were genetically modified by combining the cells with the specific recombinant adenovirus ( $1 \times 10^9$  pfu) and 2 µg of lipofectamine (Gibco BRL, USA) in 100 µl RPMI.<sup>23</sup> Briefly, after 15 min of incubation at room temperature, DC ( $1 \times 10^6$  cells) were mixed with the adenoviral-lipofectamine complex to a final MOI of 1000 and incubated for a further 2 hr to allow viral adsorption. The transfected DC were incubated for a further 48 hr to establish gene transduction prior to use in the MLR as stimulators.

## RESULTS

### Expression and secretion of CTLA4<sub>e</sub>EGFP by genetically modified cells

The expression of the transferred CTLA4<sub>e</sub>EGFP gene into CHO cells (data not shown) and fibroblasts (Fig. 1) was illustrated by detection of green fluorescent cells under UV illumination. Of particular note was that the ADV-EGFP transduced fibroblasts demonstrated stronger nuclear and cytoplasmic localization of the EGFP native protein in



**Figure 1.** CTLA4<sub>e</sub>EGFP expression by genetically modified CHO cells and fibroblasts. Autoradiographs showing immunoprecipitation with an anti-GFP mAb of <sup>35</sup>S-methionine/cysteine-labelled proteins from CHO cells (a and b) transfected with povCTLA4<sub>e</sub>EGFP, phuCTLA4<sub>e</sub>EGFP or pEGFP-N1 plasmid DNA and ovine dermal fibroblasts (c) infected with recombinant adenoviral particles containing the ADVovCTLA4<sub>e</sub>EGFP or ADV-EGFP gene constructs. Immunoprecipitates were subject to electrophoresis in 12.5% SDS-PAGE gels under non-reduced (a) and (c) or reduced (b) conditions. (d) Expression of green fluorescence by adenoviral-transduced fibroblasts under UV-microscopy (magnification  $\times 20$ ). Upper panel (d) shows transduction with ADV-EGFP and lower panel (d) with ADVovCTLA4<sub>e</sub>EGFP. The autofluorescence of untransfected cells (not shown) was negligible.

contrast to the diffuse cytoplasmic staining observed with those cells modified genetically with ADVovCTLA4<sub>e</sub>EGFP (Fig. 1d). The differential fluorescent intensity between these cells was also marked by a higher mean-fluorescent intensity reading for the ADV-EGFP-infected fibroblasts (data not shown). These genetically modified cells were shown to secrete ovine and human CTLA4<sub>e</sub>EGFP fusion proteins by immunoprecipitation of the conditioned media with anti-GFP mAbs (Fig. 1). The conditioned medium from CHO cells transfected with pEGFP-N1 and fibroblasts infected with ADV-EGFP were used as controls for EGFP synthesis. The autoradiographs of the immunoprecipitates obtained from cells treated with both control vectors demonstrated a distinct 30 kDa band corresponding to EGFP, while cells transfected with povCTLA4<sub>e</sub>EGFP and phuCTLA4<sub>e</sub>EGFP illustrated bands corresponding to molecular sizes of 62 kDa and 53 kDa,

respectively (Fig. 1a, b, c). Higher molecular size bands of 120 kDa and 110 kDa for ovine and human CTLA4<sub>e</sub>EGFP, respectively, were observed only in non-reduced SDS-PAGE gels indicating the formation of homodimers (Fig. 1a). The immunoprecipitation of conditioned medium derived from fibroblasts infected with ADVovCTLA4<sub>e</sub>EGFP confirmed the secretion of the ovine molecule by the detection of bands corresponding to both molecular species (62 kDa and 120 kDa) on non-reduced gels (Fig. 1c).

#### Characterization of ovine DC derived from pseudo-afferent lymphatic cannulation

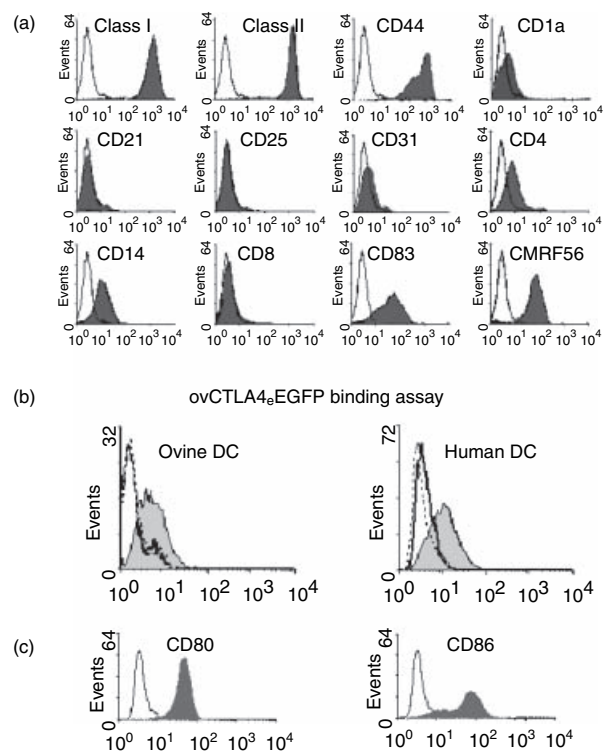
Lymphatic cells obtained from the cannulation of the pre-femoral lymph duct consisted of 70–80% of cells with typical dendritic cell morphology upon enrichment with metrizamide density centrifugation. The profiles of DC phenotypic markers were examined on cells that were obtained from the lymphatic drainage for up to 14 days postcannulation. Figure 2(a) shows the profile of DC obtained 8 days postcannulation with high levels of MHC class I, II, CD83, CD44 and CMRF-56 expression. However, while these cells showed low expression of CD1a, CD4 and CD14, the markers CD8, CD21, CD25 and CD31 were undetected. The observations of these DC profiles were typically observed in three different lymphatic cannulations and showed stability in the pattern of the expressed markers with the exception of CMRF-56, which showed a variable profile between animals.

#### Binding of ovCTLA4<sub>e</sub>EGFP to ovine and human DC

The ovCTLA4<sub>e</sub>EGFP fusion protein obtained from fibroblast infected with ADVovCTLA4<sub>e</sub>EGFP was quantitated by ELISA and used at a concentration equivalent to 20 µg/ml of EGFP to assess the binding activity to both human and ovine DC. While the human monocyte-derived DC were confirmed by flow cytometry to express the CTLA4 receptors CD80/86 (Fig. 2c) this was not established for the ovine cells due to the unavailability of ovine specific mAbs. Detection of ovCTLA4<sub>e</sub>EGFP with anti-GFP mAb demonstrated binding to both ovine and human DC as shown by an increased shift in fluorescence compared to the isotype-matched X63 control (Fig. 2b). In contrast the EGFP native protein, as a negative control, showed no reactivity (data not shown). Furthermore, the specificity of the ovCTLA4<sub>e</sub>EGFP binding to CD80/86 on both human and ovine DC was confirmed by blockade with recombinant human CTLA4-Ig. Interestingly, the binding pattern of ovCTLA4<sub>e</sub>EGFP mirrored the expression of CD86 in the human DC.

#### ovCTLA4<sub>e</sub>EGFP inhibits the two-way MLR

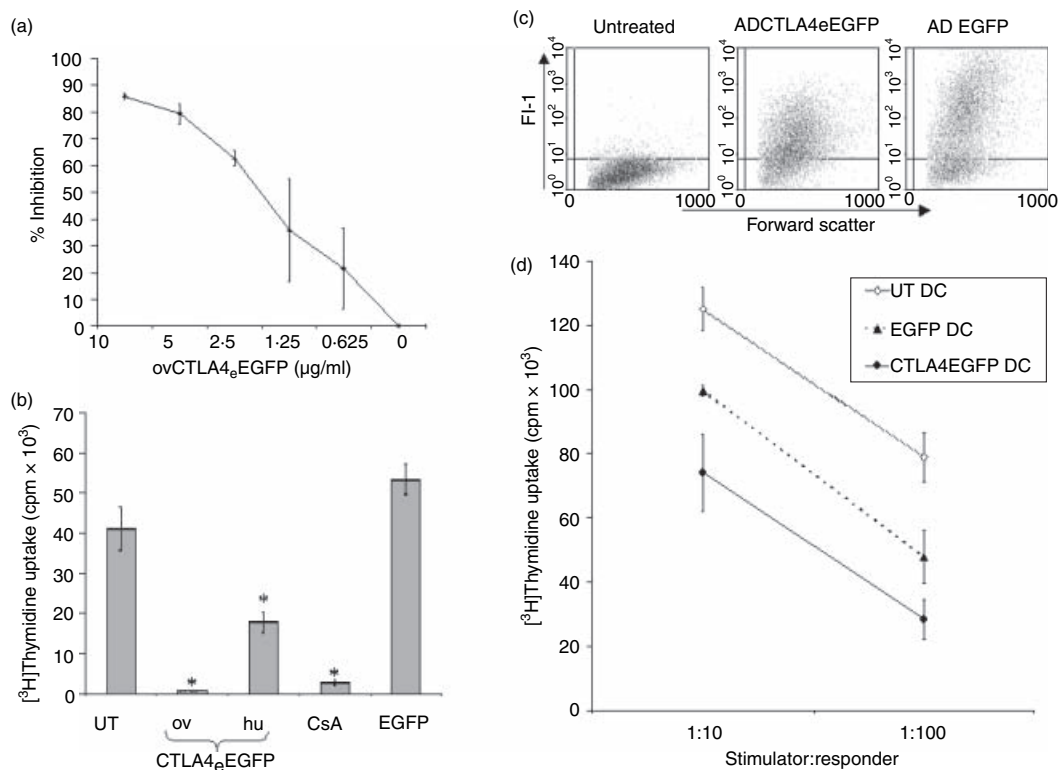
Ovine CTLA4<sub>e</sub>EGFP and EGFP derived from adenoviral-vector infected fibroblasts were quantified by ELISA and titrated for alloinhibitory activity in a two-way MLR in a concentration range of 0.625–10 µg/ml (Fig. 3a). Optimal



**Figure 2.** Phenotypic analyses of ovine lymphatic dendritic cells and the binding activity of ovCTLA4<sub>e</sub>EGFP by flow cytometry (a) Ovine DC obtained by pseudo-afferent cannulation of the pre-femoral lymph node were stained with control isotype antibodies (open histograms) and a panel of cell-surface antigen markers (filled histograms). Each histogram is representative of at least three different experiments. (b) Human or ovine DC were incubated with ovCTLA4<sub>e</sub>EGFP derived from recombinant adenovirus modified fibroblasts. The bound ovCTLA4<sub>e</sub>EGFP was detected with an anti-GFP mAb as shown in the shaded histogram and the negative-isotype matched mAb (X63) represented by the solid line. The dotted line represents blockade of ovCTLA4<sub>e</sub>EGFP binding with CTLA4-Ig. (c) Human DC were stained with anti-CD80 and anti-CD86 mAbs to confirm the presence of the CTLA-4 receptors. Flow cytometric analysis of CD80 and CD86 staining is represented by the shaded histogram with X63 represented by the unshaded histogram.

inhibition (80–90%) compared to the untreated MLR was demonstrated in a concentration range of 5–10 µg/ml equivalence to native EGFP.

The comparative inhibitory effects of ovCTLA4<sub>e</sub>EGFP and huCTLA4<sub>e</sub>EGFP were also tested in the ovine MLR. The histogram in Fig. 3(b) demonstrated a 98% ( $P = 0.012$ ) and 65% ( $P = 0.033$ ) inhibition of the ovine MLR, by the ovine and human fusion proteins, respectively, at a concentration of 5 µg/ml. The observed inhibition of the ovine MLR by the ovCTLA4<sub>e</sub>EGFP was comparable to the effects of CsA (100 ng/ml), which demonstrated 93% ( $P = 0.0076$ ) inhibition. The EGFP derived from ADV-EGFP modified cells at a concentration of 2.5–5 µg/ml demonstrated a modest allostimulatory effect compared to the untreated MLR.



**Figure 3.** Alloinhibitory properties of ovCTLA4<sub>e</sub>EGFP. (a) Dose–response inhibition of a two-way ovine MLR by ovCTLA4<sub>e</sub>EGFP. Responses are expressed as the percentage of inhibition relative to the untreated MLR. (b) Comparative alloinhibitory properties of ovCTLA4<sub>e</sub>EGFP, huCTLA4<sub>e</sub>EGFP and native EGFP. Fusion proteins were normalized to 5 μg/ml of EGFP and added to an ovine MLR. CsA was added at 100 ng/ml as an established inhibitory control. (c) Ovine DC were transfected with ADVovCTLA4<sub>e</sub>EGFP or ADV-EGFP. Cell transduction efficiency is demonstrated as a dot-plot representation (FL-1 versus forward scatter). (d) DC modified with ADVovCTLA4<sub>e</sub>EGFP were used as stimulators of ovine PBMC at ratios of 1:10 and 1:100. Unmodified DC and DC modified with ADV-EGFP were used as controls. Each treatment group consisted of triplicates and each graph is representative of three different experiments. \**P* < 0.05, unpaired Student's *t*-test.

#### ADVovCTLA4<sub>e</sub>EGFP transfected DC inhibit allostimulation

In this set of experiments ovine DC were transfected with ADVovCTLA4<sub>e</sub>EGFP and compared against cells transfected with ADV-EGFP as a control. Fluorescence resulting from the EGFP-tag in transfected DC was evaluated for both the native and fusion protein by flow cytometry (Fig. 3c). While both transfectants showed an equivalent level of transfection (70%), the ADV-EGFP transfected DC displayed a higher EGFP fluorescence as revealed by an MFI of 200 in contrast to an MFI of 50 for ADVovCTLA4<sub>e</sub>EGFP transfectants.

In the DC-MLR at stimulator:responder ratios of 1:10 and 1:100 the ADVovCTLA4<sub>e</sub>EGFP transfected DC demonstrated an inhibition of 41% (*P* = 0.003) and 64% (*P* = 0.003) compared to the unmodified DC, respectively. Surprisingly, in contrast to the two-way MLR, ADV-EGFP transfected DC also inhibited the MLR but to a lesser extent than ADVovCTLA4<sub>e</sub>EGFP transfected DC (Fig. 3d). Similar results were obtained with human transfected DC (data not shown). To clarify the observed

immunomodulation by ADVovCTLA4<sub>e</sub>EGFP, phenotypic analysis was confined to human DC due to the lack of availability of ovine reactive mAbs (data not shown). These analyses revealed that ADVovCTLA4<sub>e</sub>EGFP transfection showed a modest up-regulation of CD40 expression on DC whereas ADV-EGFP transfection resulted in a 15–20% down-regulation. However, both constructs produced only modest increases in the expression of MHC class II, CD86 and CD83, which is consistent with previously reported effects mediated by adenoviral transfection.<sup>8</sup>

#### DISCUSSION

This study reports the generation and characterization of a fusion protein consisting of CTLA4 uniquely tagged with EGFP. Of important relevance to this study is the preparation of an ovine CTLA4 construct in a recombinant adenoviral vector to be used in a gene therapy strategy in a large animal model of allograft transplantation. The sheep model of renal transplantation established in this laboratory<sup>13</sup> provides an inexpensive approach to study the



intervention of allograft rejection compared to primate models and is clinically more relevant than murine and rodent models.

The proposed strategy for use in the ovine model to improve allograft outcome will utilize genetically modified dendritic cells. With respect to this approach we have obtained dendritic cells by the cannulation of the prefemoral lymph nodes of sheep. In contrast to the human or murine situation the feasibility of generating ovine DC from peripheral blood monocyte precursors was restricted by the availability of ovine-specific GM-CSF and IL-4. The study by Blacklaws<sup>24</sup> has attempted to generate ovine DC from monocytes, although the efficiency of obtaining large numbers for an experimental therapeutic strategy is potentially limited by poor yields. Based on our experience with lymphatic cannulation, approximately 5 million DC/day was obtained routinely with a purity of 70–80% following metrizamide gradient enrichment. The daily collection of DC and cryopreservation of cells provides a means for building up a cache for subsequent cellular therapy in a transplant setting. In addition to the earlier studies by Dandie<sup>25</sup> on ovine DC phenotypes our study provides a more definitive characterization of these cells by the cross-reactivity of mAbs to the human DC markers, CMRF-56<sup>26</sup> and CD83. Indeed, these isolated DC demonstrated other typical features, which included high MHC class II expression and the ability to provoke a strong proliferative stimulus when challenged with allogeneic lymphocytes. Additional phenotypic characteristics were revealed by the binding of ovCTLA4<sub>e</sub>EGFP fusion protein to ovine DC, which recognizes the expression of CD80/86 on these cells.

Prior to the studies on the genetic modification of ovine DC with ADVovCTLA4<sub>e</sub>EGFP, the structural and functional characteristics of ovCTLA4<sub>e</sub>EGFP were confirmed by comparisons with its human homologue. Following transfection of CHO cells, immunoprecipitation of conditioned media from transfected cells indicated that CTLA4<sub>e</sub>EGFP is secreted predominantly in a monomeric form, although there is a minor proportion of high molecular species identified only in non-reducing SDS-PAGE, which is indicative of sulphhydryl protein interaction and dimer formation. It is plausible that the observed higher molecular size for the ovine fusion protein reported in Fig. 1(a) is due to post-translational modification of the CTLA4<sub>e</sub>EGFP and may be attributed to an extra N-glycosylation site and three O-glycosylation motifs identified in the ovine gene sequence<sup>15</sup> compared to the human. These structural differences did not appear to influence the ability of CTLA4<sub>e</sub>EGFP to bind to its target cells or to inhibit the MLR. The cross-species binding of ovCTLA4<sub>e</sub>EGFP to human DC is related to the highly conserved MYPPPY motif, which facilitates strong binding to CD80/86.<sup>27</sup> Furthermore, the inhibitory activity of CTLA4<sub>e</sub>EGFP paralleled that reported for CTLA4-Ig<sup>28</sup> at comparable concentrations of 1–10 µg/ml.

The confirmation of CTLA4<sub>e</sub>EGFP function will allow further studies on the direct genetic modification of DC. While dendritic cells transfected with adenoviral vectors

containing the green fluorescent protein as a reporter molecule to monitor the transfection efficiency of DC have been used previously,<sup>29</sup> our studies have combined EGFP to a functional immunomodulatory molecule and reported *in vitro* function. A similar study by Zawitkowski<sup>30</sup> has shown *in vitro* function of the CD40 molecule fused in-frame with EGFP. In our studies the EGFP tag facilitated the flow cytometric detection of transfected DC expressing functional CTLA4 and routinely demonstrated a transduction efficiency of 70–90%. In addition, the potential of obtaining even higher percentages of CTLA4<sub>e</sub>EGFP expressing cells may be achieved by flow sorting the genetically modified DC. In our studies the CTLA4<sub>e</sub>EGFP expressing DC showed a significantly reduced capability of inducing the proliferation of allogeneic T cells compared to the unmodified or ADV-EGFP transfected DC, thus demonstrating potential for use in a transplant setting to modify allograft outcome.

In summary, this study verified that the ovine CTLA4 fusion protein functions in a similar manner to its human homologue and demonstrated cross-species reactivity. These results warrant further investigation into the effects of DC transduced with adenoviral CTLA4<sub>e</sub>EGFP *in vivo* in an ovine model of renal transplantation.

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